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Differences in DNA Binding Specificity among *Roseolovirus* Origin Binding Proteins

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The *Roseolovirus* genus of the *Betaherpesvirinae* consists of the very closely related viruses, *human herpesvirus 6* variants A and B (HHV-6A and HHV-6B) plus the somewhat more distantly related *human herpesvirus 7* (HHV-7). The roseoloviruses each encode a homolog of the alphaherpesvirus origin binding protein (OBP) which is required for lytic DNA replication. In contrast, members of the other betaherpesvirus genera, the cytomegaloviruses, initiate DNA replication by a different mechanism. To better understand the basis of roseolovirus OBP sequence specificity, we investigated their ability to recognize each other's binding sites. HHV-6A OBP (OBP_{H6A}) and HHV-6B OBP (OBP_{H6B}) each bind to both of the HHV-7 OBP sites (OBP-1 and OBP-2) with similar strengths, which are also similar to their nearly equivalent interactions with their own sites. In contrast, HHV-7 OBP (OBP_{H7}) had a gradient of binding preferences: HHV-7 OBP-2 > HHV-6 OBP-2 > HHV-7 OBP-1 > HHV-6 OBP-1. Thus, the roseolovirus OBPs are not equally reciprocal in their recognition of each other's OBP sites, suggesting that the sequence requirements for the interaction of OBPH7 at the OBP sites in its cognate *ori*Lyt differ from those of OBPH6A and OBPH6B. © 2001 Academic Press

Key Words: human herpesvirus 6; HHV-6; *human herpesvirus 7*; HHV-7; origin binding protein; DNA replication initiation; herpesvirus.

INTRODUCTION

Two general mechanisms for initiating lytic DNA replication of herpesviruses have been identified among the alpha-, beta-, and gammaherpesvirus subfamilies. Epstein–Barr virus, a member of the *Gammaherpesvirinae,* relies on transcription factors to recruit the replication machinery to its origin of lytic replication (*ori*Lyt) (Baumann *et al.,* 1999). In contrast, members of the *Alphaherpesvirinae*, such as herpes simplex virus type 1 (HSV-1), encode proteins that interact in a sequence-specific manner with their *ori*Lyts to initiate replication (origin binding proteins, or OBP) (reviewed in Boehmer and Lehman, 1997). In the betaherpesviruses, there is a schism between the cytomegaloviruses and the roseoloviruses. The cytomegaloviruses do not encode an OBP homolog and have complex *ori*Lyts with features in common with gammaherpesvirus *ori*Lyts (Anders *et al.,* 1992; Baumann *et al.,* 1999). Roseoloviruses [*Human herpesviruses 6* variants A and B (HHV-6A and HHV-6B) and *Human herpesvirus 7* (HHV-7)] encode OBPs and have *ori*Lyt structures that are remarkably similar to those of the alphaherpesviruses (Inoue *et al.,* 1994; Lawrence *et al.,* 1995; van Loon *et al.,* 1997).

The best characterized OBP is that of HSV-1 (OBP_{H1}). OBP_{H1}, encoded by gene UL9, likely binds as a dimer at each of its two high-affinity recognition sites, Box I and Box II, in ori_S (Elias and Lehman, 1988; Lee and Lehman, 1999). Interactions with the viral single-stranded DNA binding protein (ICP8) enhance OBP_{H1} helicase activity and lead to local unwinding followed by the recruitment of the viral replication machinery (Boehmer and Lehman, 1997). Similarly, the OBPs of HHV-6B (OBP_{H6B}) and HHV-7 (OBP_{H7}) each bind two OBP sites (OBP-1 and OBP-2) that flank an AT-rich region in their *ori*Lyts (Inoue *et al.,* 1994; Krug *et al.,* 2001) (Fig. 1).

Analyses of reciprocal interactions among herpesvirus OBPs have been useful in defining the link between similarity in their carboxyl-terminal DNA binding domains and conservation of sequence specificity. Among alphaherpesviruses, the OBP_{H1} DNA binding domain (amino acids 564–832) is 38% identical and 49% similar to that of varicella zoster virus (OBP_{VZ}) (amino acids 551–813) (Deb and Deb, 1991; Chen and Olivo, 1994). Despite their divergence, both recognize identical 11-bp sequences that are present in their *ori*Lyts (Stow *et al.,* 1990; Hazuda *et al.,* 1991). Nonetheless, OBP_{VZ} can only partially complement the replication of an HSV-1 UL9-null mutant (Chen *et al.,* 1995). The DNA binding domains of OBP_{H1} and OBP_{H6B} (amino acids 482–770) are only 20% identical and 32% similar (Inoue and Pellett, 1995). The proteins have diverged such that their consensus recognition sequences differ (Koff and Tegtmeyer, 1988; Deb and

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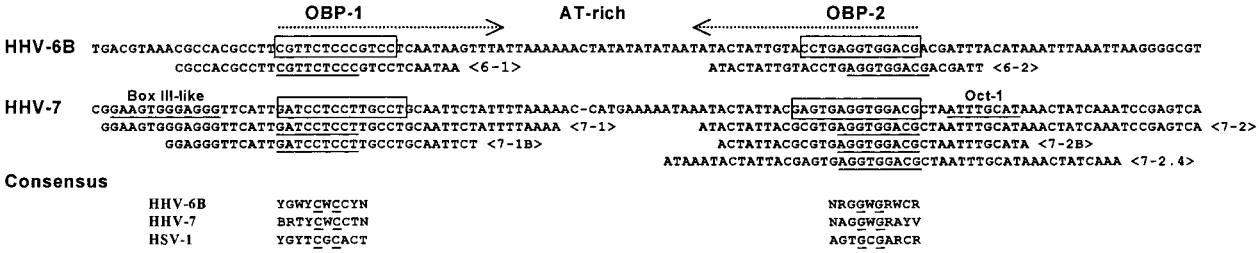

FIG. 1. OBP sites in the *ori*Lyt regions of HHV-6B and HHV-7. The minimal recognition sequences are boxed and the OBP-1 and OBP-2 cores of each are underlined (Inoue and Pellett, 1995; Krug *et al.,* 2001). Areas of the OBP sites and AT-rich spacer element that contain imperfect dyad symmetry are indicated by arrows. A Box III-like element previously reported for the HHV-7 *ori*Lyt is underlined (Krug *et al.,* 2001). A potential Oct-1 site (ATGCAAAT) is underlined (van Loon *et al.,* 1997); the complementary strand is shown. Oligonucleotides used in this study are below each *ori*Lyt sequence. In the alignment of the consensus recognition sequence determined for OBP_{H6B}, OBP_{H7}, and OBP_{H1}, shared residues are underlined.

Deb, 1989; Weir and Stow, 1990; Inoue and Pellett, 1995) (Fig. 1) and they are unable to recognize each other's binding site (Inoue *et al.,* 1994; Inoue and Pellett, 1995).

Among the roseoloviruses, the OBP_{H6B} and OBP_{H7} DNA binding domains are 57% identical and 69% similar. The 9-bp consensus recognition sequences experimentally determined for OBP_{H6B} and OBP_{H7} are identical at five positions, defining YCWCC (Y, T, or C and W, T, or A) as the core of the roseolovirus OBP recognition sequences (Inoue and Pellett, 1995; Krug *et al.,* 2001) (Fig. 1). While their 9-bp OBP-2 sites are identical, their OBP-1 sites differ at five positions, some of which result in mismatches with their counterpart's consensus sequence (Fig. 1). In contrast to OBP_{H6B}, which binds its two sites comparably (Inoue *et al.,* 1994), OBP_{H7} binds more strongly to its OBP-2 site than its OBP-1 site (Krug *et al.,* 2001). Thus, although the roseolovirus OBPs share some DNA binding properties, the details of their interactions with their cognate *ori*Lyts may differ. Consistent with this, in an examination of reciprocal interactions in transient replication assays, HHV-7-infected cells replicated an HHV-6 *ori*Lyt-containing plasmid, but not vice versa (van Loon *et al.,* 1997).

In this study, we biochemically evaluated the cross-recognition of roseolovirus OBP sites by their OBPs to determine whether the OBPs from these closely related viruses are functionally interchangeable or if the lack of full reciprocity between the roseoloviruses at the level of DNA replication is due to differences in their interactions with the OBP sites. We also compared the sequences of the DNA binding domains of these closely related, yet distinct, OBPs in the context of other herpesvirus OBPs to illuminate the structural basis for their sequence specificities and thereby extend our understanding of OBP–DNA interactions.

RESULTS

In vitro expression of OBP_{H6A}, OBP_{H6B}, and OBP_{H7}

We previously found that as for OBP_{H1}, *in vitro* expressed OBP_{H6B} has DNA binding properties very similar to those of protein purified from a bacterial expression system (Inoue and Pellett, 1995). Full-length or carboxyl-terminal segments of OBP_{H6A}, OBP_{H6B}, and OBP_{H7} were expressed in coupled *in vitro* transcription–translation reactions (IVTT) and separated in a polyacrylamide gel. Comparable amounts of ^{35}S-labeled protein were detected (Fig. 2). The carboxyl-terminal segments (tOBP) correspond to OBP_{H6B} subdomains A and B (amino acids 482–770), which are together required for DNA binding activity in electrophoretic mobility shift analyses (EMSA) (Inoue and Pellett, 1995): OBP_{H6A} amino acids 482–780, OBP_{H6B} amino acids 482–780, and OB_{PH7} amino acids 484–787. As previously observed for OBP_{H6B}, urea-containing loading buffer was required to disrupt aggregates and allows full-length OBP_{H6A} and OBP_{H7} to enter the gel (Fig. 2, compare lanes 1 through 3 to lanes 8 through 10); the carboxyl-terminal segments did not form such aggre-

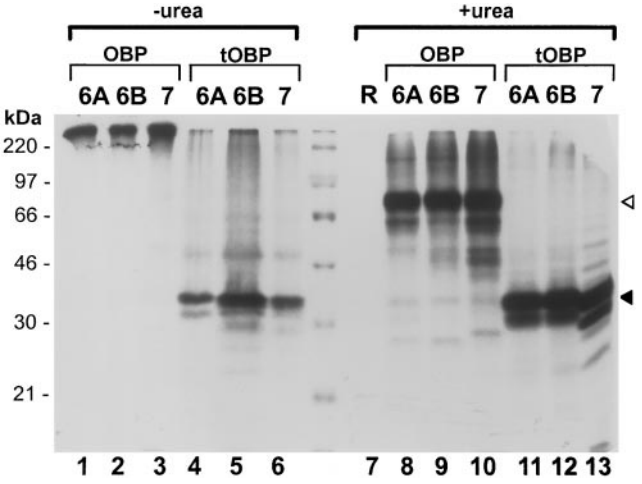

FIG. 2. *In vitro* expression of OBP_{H6A}, OBP_{H6B}, and OBP_{H7}. Full-length (open arrowhead, approximately 89 kDa) and carboxyl-terminal truncated portions of OBP (tOBP, closed arrowhead, approximately 36 kDa) expressed in IVTT from pcDNA3 constructs with a Kozak translation initiation sequence. ^{35}S-labeled products were mixed with an equal volume of loading buffer without (lanes 1–6) or with 6 M urea (lanes 7–13) and analyzed by SDS–PAGE. Lane 7 contains negative control lysate programmed with a plasmid that contains HHV-7 U73 in the reverse orientation (R).

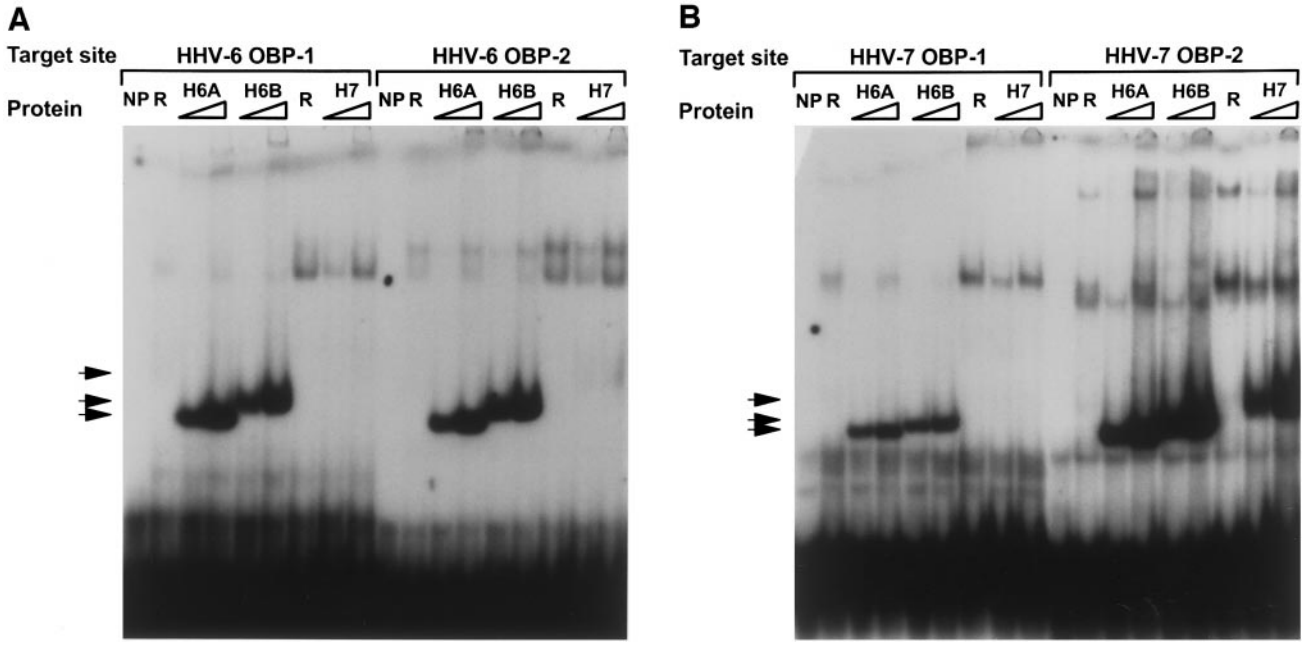

FIG. 3. Examination of reciprocity between OBPs and OBP sites of HHV-6A, -6B, and -7 by EMSA. ^{32}P-labeled DNA duplexes containing HHV-6 OBP-1 and OBP-2 (oligonucleotides 6-1 and 6-2, respectively) in A and duplexes containing HHV-7 OBP-1 and OBP-2 (oligonucleotides 7-1 and 7-2, respectively) in B were incubated with 1.0 and 2.5 μl of IVTT lysates containing $tOBP_{H6A}$ and $tOBP_{H6B}$ in buffer B and $tOBP_{H7}$ in buffer A, or 2.5 μl of negative control lysate described in Fig. 2 legend (R). NP, no lysate added. The upper, middle, and lower arrowheads in each panel point to specific mobility shifts generated by $tOBP_{H7}$, $tOBP_{H6B}$, and $tOBP_{H6A}$, respectively.

gates (lanes 4 through 6). The insolubility of the full-length molecules precluded their use in binding studies. The soluble carboxyl-terminal portions were used in the remainder of the experiments described here.

Reciprocal interactions

There are limits on the use of OBP consensus recognition sequences in predicting the strength of their interactions with a given sequence. This is because sequences flanking the 9-bp core can influence recognition (Inoue and Pellett, 1995; Krug *et al.,* 2001) and the consensus sequences were determined based on single base-pair changes relative to the native binding sequence. An accumulation of several individually permissive, but less than optimal, changes could result in a consensus-conforming sequence that is not efficiently recognized. Among the roseoloviruses, there are examples of both types of situations: the 9-bp OBP-2 sites are identical between HHV-6 and HHV-7 *ori*Lyts, but their flanking sequences differ; and the 9-bp HHV-6 and HHV-7 OBP-1 sites differ at one and two positions, respectively, from each other's consensus (Fig. 1). Thus, we examined the ability of each roseolovirus OBP to bind each other's native OBP site in the context of the native flanking sequences.

Two types of EMSA experiments were done: direct binding to labeled oligonucleotides (Fig. 3) and assessment of binding strength by competition with unlabeled oligonucleotides (Fig. 4). Two binding buffers were used that differ in their concentrations of NaCl and $MgCl_2$. Unless stated otherwise, binding reactions were done in buffer A for $tOBP_{H7}$ (Krug *et al.,* 2001) and buffer B for $tOBP_{H6A}$ and $tOBP_{H6B}$ (Inoue *et al.,* 1994). Specificity was demonstrated by supershifting complexes with specific antibodies, as was done previously for $tOBP_{H7}$ (Krug *et al.,* 2001) (data not shown).

To set the stage for the reciprocity experiments, we did several experiments that confirmed and somewhat extended previous observations of the interactions of the roseolovirus OBPs with their cognate sites. As previously shown for OBP_{H6B}, $tOBP_{H6A}$ and $tOBP_{H6B}$ bound strongly to oligonucleotides containing both of their homologous OBP sites (oligonucleotides 6-1 and 6-2, respectively) (Figs. 3A and 4A). The slight difference in the mobility of the complexes might be attributed to a small difference in the net charges of the proteins. While this is the first biochemical description of OBP_{H6A} DNA binding activity, these results were not unexpected, given that OBP_{H6A} and OBP_{H6B} share 97.6% amino acid identity and 98.6% amino acid similarity (Fig. 6) and were interchangeable in *ori*Lyt transient replication assays between HHV-6A- and HHV-6B-infected cells (Dewhurst *et al.,* 1994).

In experiments similar to those previously described (Krug *et al.,* 2001), $tOBP_{H7}$ strongly recognized an HHV-7 OBP-2-containing oligonucleotide (7-2) in buffer A, but did not interact with an oligonucleotide (7-1) that con-

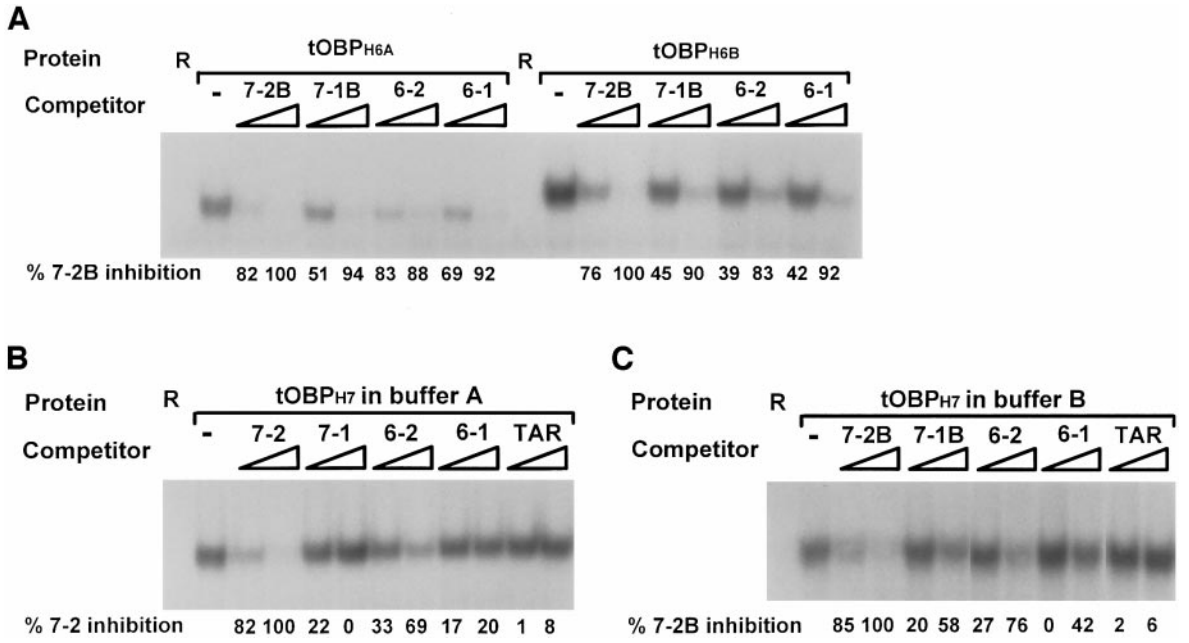

FIG. 4. Reciprocity analyzed by competitive EMSA. (A) Competition of oligonucleotides containing the four OBP sites with ^{32}P-7-2 for binding to $tOBP_{H6A}$ and $tOBP_{H6B}$. Sixteen- and 80-fold molar excesses of DNA duplexes containing HHV-7 OBP-2 and OBP-1 (oligonucleotides 7-2B and 7-1B, respectively) and DNA duplexes containing HHV-6 OBP-2 and OBP-1 (oligonucleotides 6-2 and 6-1, respectively) were incubated with 1.0 μl of IVTT lysate containing $tOBP_{H6A}$, $tOBP_{H6B}$, or control lysate (R) in buffer B. The percentage of binding inhibition to labeled 7-2 DNA by each competitor DNA relative to binding inhibition with 80-fold molar excesses of 7-2B competitor is shown beneath the gel. (B and C) As for A, except 2.0 μl of IVTT lysate containing $tOBP_{H7}$ was reacted with DNA in buffer A and buffer B, respectively. Sixteen- and 80-fold molar excesses of DNA duplexes containing HHV-7 OBP-2 and OBP-1 (oligonucleotides 7-2 or 7-2B and 7-1 or 7-1B, respectively) and DNA duplexes containing HHV-6 OBP-2 and OBP-1 (oligonucleotides 6-2 and 6-1, respectively) were used as competitors. The percentage of binding inhibition to labeled 7-2.4 (B) or 7-2 (C) DNA by each competitor DNA relative to the binding inhibition with 80-fold molar excesses of 7-2 or 7-2B competitor is shown beneath the gel. Oligonucleotides 7-2 and 7-2B both contain the HHV-7 OBP-2 site (Fig. 1) and have comparable affinities for $tOBP_{H7}$ (Krug *et al.,* 2001). TAR, negative control oligonucleotide with no matches to the OBP_{H6B} or OBP_{H7} consensus sequence.

tains the HHV-7 OBP-1 site (Fig. 3B, upper arrow). In addition, although $tOBP_{H7}$ binding to its high-affinity site (OBP-2) is greater in buffer A than in buffer B, a significant (greater than 30% of wild-type inhibition) dose-dependent interaction of $tOBP_{H7}$ with its lower affinity site (OBP-1) was detected only in buffer B (Figs. 4B and 4C). This reactivity was not due to a loss of specificity since the TAR oligonucleotide that contains no OBP site did not compete in either buffer condition.

The key experiments presented here involve examination of the ability of the roseolovirus OBPs to interact with each other's OBP sites. In the direct binding experiments, the HHV-6 OBPs bound strongly to both HHV-7 OBP sites (Fig. 3B). Likewise, in the competition experiments, the strength of their interactions with oligonucleotides containing the HHV-7 OBP sites were comparable to those with their cognate OBP sites (Fig. 4A). In contrast, $tOBP_{H7}$ interacted weakly with the HHV-6 OBP-2 target (oligonucleotide 6-2) (Fig. 3A, upper arrow) and did not complex with the HHV-6 OBP-1-containing oligonucleotide (6-1). Although reactivity of $tOBP_{H7}$ with HHV-6 OBP-1 was not detected in the direct binding experiment, in the competition experiment, a weak interaction was detected in buffer B (Fig. 4C). In both reaction buffers, binding to $tOBP_{H7}$ was more effectively competed by oligonucleotides containing the HHV-7 OBP-2 site (7-2 and 7-2B) than one containing the HHV-6 OBP-2 site (6-2).

To summarize the results shown in Figs. 3 and 4, in contrast to the strong recognition of both HHV-7 OBP sites by $tOBP_{H6A}$ and $tOBP_{H6B}$, $tOBP_{H7}$ had preferential interactions for OBP-2 site-containing oligonucleotides as compared to oligonucleotides containing the OBP-1 site (HHV-7 OBP-2 > HHV-6 OBP-2 > HHV-7 OBP-1 > HHV-6 OBP-1).

Influence of the OBP core sequence on OBP_{H7} recognition

To assess whether the observed $tOBP_{H7}$ gradient of interactions was due to differences in either the core binding region or the flanking sequences, we constructed three 22-bp oligonucleotides that contained 10-bp core sequences from HHV-7 OBP-1, HHV-6 OBP-1, and OBP-2 (identical between HHV-6 and HHV-7), each flanked on both sides by the 6-bp sequence that surrounds the native HHV-7 OBP-2 site (Fig. 5). Parenthetically, although the core sequence used in these oligo-

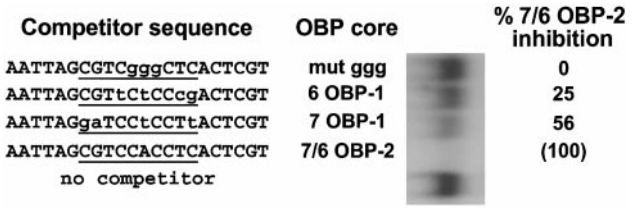

FIG. 5. Effect of OBP core sequence on $tOBP_{H7}$ recognition. Competition of oligonucleotides containing the three different OBP sites surrounded by the native HHV-7 OBP-2 flanking sequences. ^{32}P-7-2B was used for binding to $tOBP_{H7}$. Eighty-fold molar excesses of DNA duplexes containing 10-bp of the OBP-2 site that is identical between HHV-6 and HHV-7 (7/6 OBP-2), the HHV-7 OBP-1 (seven OBP-1), or the HHV-6 OBP-1 (six OBP-1) sequences were incubated with 2.0 μl of IVTT lysate containing $tOBP_{H7}$ in buffer C. The sense-strand sequences of the competitors are shown. The 10-bp sequence of the OBP sites is underlined. Differences with the OBP-2 sequence are in lowercase. The percentage of binding inhibition to labeled 7-2B DNA by each competitor DNA relative to that of 80-fold molar excesses of 7/6 OBP-2 competitor is shown. "mut ggg," mutant oligonucleotide that has GGG in place of the central CAC sequence. This mutation has been previously demonstrated to be nonpermissive both for $tOBP_{H7}$ recognition and for HHV-7 *ori*Lyt-mediated transient replication (van Loon *et al.,* 1997; Krug *et al.,* 2001).

nucleotide competitors was one base-pair longer than the 9-bp minimal core sequence, in previous experiments, the substitution of any base at this tenth position did not greatly influence OBP_{H6B} and OBP_{H7} recognition (Inoue and Pellett, 1995; Krug *et al.,* 2001). In competitions with 80-fold molar excesses of these oligonucleotides, the order of OBP site preferences for $tOBP_{H7}$ was the same as described above for each of the sites surrounded by their native flanking sequences. This result indicates that the core binding sequences exert the major effect on $tOBP_{H7}$ binding specificity. Nonetheless, the difference in $tOBP_{H7}$ binding to oligonucleotides containing the identical OBP-2 sites of HHV-6 and HHV-7 in the context of their divergent native flanking sequences (Figs. 3 and 4) indicates that sequences flanking the core influence to a small degree the efficiency of $tOBP_{H7}$ recognition.

Previously we found a gradient of strong to weak recognition by $tOBP_{H7}$ with a panel of oligonucleotides that contain combinations of substitutions in the HHV-7 OBP-2 site that make it progressively more like an OBP-1 site (Krug *et al.,* 2001). Based on this, we hypothesize that no single base difference between the cores of the OBP-1 sites is entirely responsible for the difference in the strength of their interaction with $tOBP_{H7}$.

Comparison of roseolovirus and alphaherpesvirus OBP amino acid sequences

To identify regions of high-sequence conservation or divergence that may relate to sequence specificity, we aligned the carboxyl-terminal DNA-binding domain sequences of 12 herpesvirus OBPs (Fig. 6). As previously described, the DNA-binding domain of OBP_{H6B} has two subdomains, one that directly interacts with DNA (subdomain B) and another that modulates this activity (subdomain A) (Inoue and Pellett, 1995). Within subdomain A, we identified one segment that is more highly conserved among the alphaherpesviruses (alpha A′ or "box I", Wu *et al.,* 1996) and another segment that is relatively highly conserved among the roseoloviruses (roseolo A′). Within subdomain B, a 75 amino acid segment (OBP B′) is the most highly conserved region among the herpesvirus OBPs, as previously noted in smaller comparisons (Martin *et al.,* 1994; Inoue and Pellett, 1995).

Subdomain B is more highly conserved than subdomain A within and between members of the alphaherpesvirus and roseolovirus OBPs. In the alphaherpesviruses, OBP_{H1} and OBP_{VZ} are 45% identical at the amino acid level in subdomain B and 30% identical in subdomain A. For the roseoloviruses, the subdomain B regions of OBP_{H6B} and OBP_{H7} share 61% amino acid identity compared to 54% identity in subdomain A. As shown by Stow *et al.* (1998), site-specific substitutions of charged residues within subdomain B of OBP_{H1} reduced or destroyed DNA binding activity; most of the critical residues identified for OBP_{H1} are conserved in the roseoloviruses (Fig. 6).

DISCUSSION

We found that the roseolovirus OBPs were not equally reciprocal in their recognition of each other's OBP sites. OBP_{H6A} and OBP_{H6B} bound to the HHV-7 OBP sites with strengths comparable to their own, but as it did with its own OBP-2 and OPB-1 sites, $tOBP_{H7}$ interacted more efficiently with HHV-6 OBP-2 than HHV-6 OBP-1. The several single-base differences with the OBP-2 core sequence in the HHV-7 and HHV-6 OBP-1 sites appear to compound to create sites that are only weakly recognized by $tOBP_{H7}$. Flanking sequences outside of the core modulate this recognition. We conclude that the sequence requirements for the interaction of OBP_{H7} at the OBP sites in its cognate *ori*Lyt differ from those for OBP_{H6A} and OBP_{H6B}.

We used *in vitro* expressed OBPs in this study. It is possible that the activity of *in vitro* expressed protein may differ from that expressed in infected cells due to differences in posttranslational modifications. However, data from studies using *in vitro* expressed OBP has been corroborated by studies utilizing OBPs expressed in bacteria (Elias *et al.,* 1992; Inoue and Pellett, 1995), baculovirus-infected cells (Koff *et al.,* 1991; Fierer and Challberg, 1995), and herpesvirus-infected cells (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988). Isler and Schaffer (2001) recently reported that OBP_{H1} does undergo phosphorylation, but this posttranslational modification did not im-

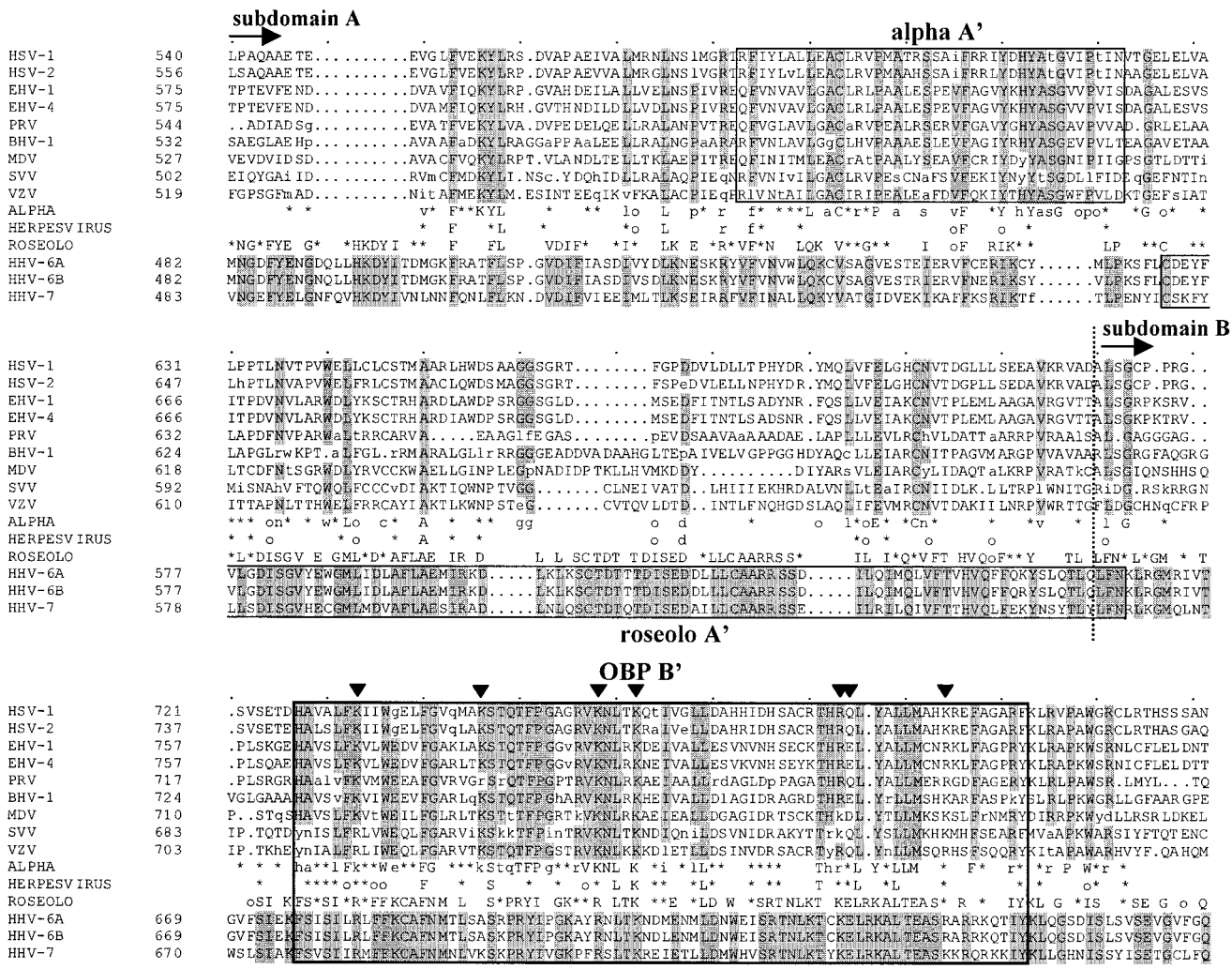

FIG. 6. Comparison of herpesvirus OBP DNA-binding domains. The amino acid sequences of OBP homologs from the alphaherpesviruses: HSV-1 (GenBank Accession No. X14112); herpes simplex virus type 2 (HSV-2, Z86099); *Equine herpesvirus 1* (EHV-1, M86664) and EHV-4 (AF030027); pseudorabies virus (PRV, *Suid herpesvirus 1*, X97257); *Bovine herpesvirus 1* (BHV-1, AJ004801); Marek's disease virus (MDV, *Gallid herpesvirus 1*, U28785); simian varicella virus (SVV, *Cercopithecine herpesvirus 7*, AF275348) and VZV (X04370); and the roseoloviruses HHV-6A (X83413), HHV-6B (AF157706), and HHV-7 (AF037218) were aligned by the Genetics Computer Group (GCG) PILEUP program (Wisconsin Package Version 10, Madison, WI) with a gap weight of eight and a gap length weight of two. The alphaherpesvirus (ALPHA) and the roseolovirus (ROSEOLO) consensus sequences are below or above their respective group of OBP sequences. In the consensus sequences, uppercase letters indicate perfect agreement in the alphaherpesviruses and roseoloviruses and lowercase letters indicate two or fewer disagreements in the alphaherpesviruses. Residues that are identical within the alphaherpesviruses or roseoloviruses are shaded. "*", indicates conservative amino acid differences in seven of the nine alphaherpesvirus or all three of the roseolovirus OBPs that are grouped (IVLM), (FYW), (RKH), (DENQ), (CSTGAP) (Henikoff and Henikoff, 1992). "o", hydrophobic grouping (IVLMFYW). Lowercase residues within an OBP sequence indicate a disagreement with the alphaherpesvirus consensus. The herpesvirus OBP consensus (HERPESVIRUS) is based on agreement between the alphaherpesvirus and roseolovirus consensus sequences. The dashed vertical line indicates the boundary between subdomains A and B determined for OBP_{H6B} (Inoue and Pellett, 1995). The degree of similarity represented in this alignment was analyzed using the GCG PLOTSIMILARITY program. Boxed areas are regions of high conservation that had similarity scores above the mean similarity score identified among the alphaherpesviruses (alpha A'), roseoloviruses (roseolo A'), or all herpesvirus OBPs (OBP B'). Arrowheads indicate residues in OBP_{H1} that were changed to alanines that resulted in a reduction of binding activity (Stow *et al.,* 1998). The figure is an extension of previously published alignments (Chen and Olivo, 1994; Inoue and Pellett, 1995; Wu *et al.,* 1996).

pact the DNA binding properties of OBP_{H1}. In addition, mutations in the HHV-6 and HHV-7 *ori*Lyt sequences that abolish binding to the *in vitro* expressed OBPs do not support the *ori*Lyt-mediated transient replication (Dewhurst *et al.,* 1994; Inoue *et al.,* 1994; van Loon *et al.,* 1997; Krug *et al.,* 2001), suggesting that the DNA-binding activities of OBPs expressed *in vitro* in large part reflect their *in vivo* function.

The ability of OBP_{H6B} to bind strongly to both HHV-7 OBP sites contrasts with the observation that the HHV-6

viral replication machinery failed to replicate an HHV-7 *ori*Lyt-containing plasmid (van Loon *et al.,* 1997). This failure indicates that either (i) additional functions of OBP_{H6} that are needed for origin activation subsequent to OBP binding are lost upon interaction with this noncognate sequence scaffold, such as local DNA bending or unwinding, or interaction with other components of the DNA replication machinery; (ii) the other components of the HHV-6 viral replication machinery cannot productively interact with the HHV-7 *ori*Lyt; or less likely, (iii) the HHV-6-infected immortalized J-Jahn cells and the HHV-7-infected primary cord blood mononuclear cells have differences in their cellular factors that affected replication efficiency. These possibilities are not mutually exclusive.

While the central structure of the roseolovirus *ori*Lyts, two OBP sites flanking a 50-bp AT-rich region, is conserved, there are differences in the origins outside this region that indicate the roseoloviruses might differ in their mechanism of DNA replication initiation. Both HHV-6A and HHV-6B *ori*Lyts contain a 3′ auxiliary region downstream from their OBP sites that contains AT-rich imperfect direct repeats of approximately 200-bp each and has thermodynamic properties consistent with a DNA unwinding element; there is no corresponding region in the HHV-7 *ori*Lyt (Dewhurst *et al.,* 1993, 1994; van Loon *et al.,* 1997). Interestingly, the HHV-7 *ori*Lyt contains two elements not present in the HHV-6 *ori*Lyt that may affect replication by HHV-6-specific factors: an element adjacent to the HHV-7 OBP-1 site that has features similar to the Box III site of the HSV-1 origins (Fig. 1), and a potential Oct-1 transcription factor binding site adjacent to the OBP-2 site. While the function of the HSV-1 Box III site is not clear, in a recent report, mutations within ori_S sequences that result in alteration of predicted stem–loop structures involving Box III were not bound by OBP_{H1} and had reduced transient replication efficiency (Aslani *et al.,* 2000). The effect of the potential Oct-1 interaction is difficult to predict, given that an Oct-1 site in an AT-rich segment of the simian virus 40 replication origin inhibits the DNA unwinding catalyzed by the viral initiator T-antigen (Kilwinski *et al.,* 1995), while host factor binding in the HSV-1 ori_S region appears to enhance DNA replication (Nguyen-Huynh and Schaffer, 1998). The roles for such sequences and structures in HHV-6 and HHV-7 replication remain to be determined.

We must bear in mind that even though the recognition of the HHV-6 OBP-1 site by $tOBP_{H7}$ is relatively weak *in vitro*, the replication of an HHV-6 *ori*Lyt-containing plasmid in HHV-7-infected cells (van Loon *et al.,* 1997) indicates that full-length OBP_{H7} can productively interact with the HHV-6 origin *in vivo*. Thus, the gradient of interactions of $tOBP_{H7}$ with the HHV-6 OBP sites and its own sites *in vitro* suggest that either (i) the interaction of OBP_{H7} with the one high-affinity site is alone sufficient for origin activation, or (ii) productive interaction of OBP_{H7} with the *ori*Lyt requires it to first bind the high-affinity site and through cooperative interactions subsequently increase the strength and stability of OBP_{H7} binding at the low-affinity site, in a manner analogous to the cooperative binding of OBP_{H1} to its sites in ori_S (Elias *et al.,* 1990).

The basis for the similarities and differences in the roseolovirus OBP DNA-binding properties resides in the amino acid sequences of subdomains A and B within their carboxyl-terminal DNA binding domains. Although subdomain B is sufficient for sequence-specific DNA interactions, subdomain A appears to increase binding specificity (Inoue and Pellett, 1995). Mutagenesis of these regions will enable further definition of the basis of OBP sequence specificity and further our understanding of the mechanism of OBP-dependent DNA replication initiation. This would contribute to the development of novel compounds that would inhibit viral replication by disrupting OBP activity (Font *et al.,* 2000) and to the design of broad-spectrum amplicon vectors containing an *ori*Lyt that could be replicated with HHV-6A, HHV-6B, or HHV-7 as helper viruses (Deng and Dewhurst, 1998; Romi *et al.,* 1999).

MATERIALS AND METHODS

Cloning and expression of OBP_{H6A}, OBP_{H6B}, and OBP_{H7}

Full-length and carboxyl terminal portions of U73 were amplified from HHV-6A strain U1102, HHV-6B strain Z29, and HHV-7 strain SB viral DNA in PCR reactions using a proofreading DNA polymerase (*Pfx*, GIBCO-BRL, Rockville, MD) and primers that contained restriction endonuclease sites and a Kozak consensus sequence surrounding the ATG. The resulting amplimers were cloned into pcDNA3 (Invitrogen, Carlsbad, CA).

OBP proteins were expressed in coupled IVTT reactions (Promega, Madison, WI) programmed with the plasmids described above in the presence of [^{35}S]methionine and separated by SDS–PAGE as previously described (Inoue *et al.,* 1994). All of the IVTT reactions in the experiments described here were programmed with the same plasmid preparation. Lysates were stored as small aliquots at −80°C and were thawed only once before use.

EMSA

Oligonucleotides were annealed, labeled, and purified as described previously (Inoue *et al.,* 1994). DNA was incubated at room temperature for 20 min in 10 μl of buffers A, B, or C with 1.0, 2.0, or 2.5 μl of programmed IVTT lysate. Buffer A consisted of 12 mM HEPES–NaOH (pH 7.6), 4 mM Tris–HCl (pH 7.6), 125 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 120 μg bovine serum albumin per ml, 12% glycerol, 5 μg salmon testes DNA per ml, and a cocktail of protease inhibitors (Com-

plete, Mini, EDTA-free, Roche). Buffer B (Inoue *et al.,* 1994) is similar to buffer A except that it contained 50 mM NaCl and no $MgCl_2$. Buffer C is similar to buffer A except that it contained 50 mM NaCl and 5 mM $MgCl_2$. DNA–protein complexes were separated in 5% polyacrylamide gels (60:1 acrylamide to *bis*-acrylamide) by using a low-ionic-strength electrophoresis buffer (Inoue *et al.,* 1994) at 4°C.

For competitive EMSA, reactions were set up as described above except that unlabeled competitor DNA was incubated with the lysate for 10 min at room temperature before the addition of the labeled target DNA. In the competition experiments, the amount of residual shifted [^{32}P]DNA in the presence of competitor was measured by phosphorimager analysis (Storm, Amersham Pharmacia, Piscataway, NJ) and expressed as the percentage of binding inhibition relative to that of the reference oligonucleotide at 80-fold molar excess. As described previously, protein interactions with unlabeled oligonucleotides that resulted in a competition that was less than 30% of the inhibition level of a control competitor were considered nonspecific (Inoue and Pellett, 1995; Krug *et al.,* 2001).

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